AD_____

Award Number: W81XWH-11-1-0041

TITLE: Breast Cancer Endothelial Cell Calcium Dynamics Using Two-Photon Microscopy

PRINCIPAL INVESTIGATOR: Javier Lapeira

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14511

REPORT DATE: January 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2012	Annual Summary	1 JAN 2011 - 31 DEC 2011

4. TITLE AND SUBTITLE
Breast Cancer Endothelial Cell Calcium Dynamics Using Two-Photon Microscopy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0041

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Javier Lapeira

E-Mail: lapeira@bme.rochester.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, NY 14511

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Control murine liver endothelial cells and murine breast tumor endothelial cells were successfully isolated and plated for the imaging and flow cytometry experiments. I have found interesting differences between normal and tumor endothelial cells well worth further studying. More specifically, I have found that the response of tumor endothelial cells to VEGF is the same regardless of whether or not they have been serum-starved. Also receptor densities of VEGFR-1 and -2 differ between normal and breast tumor ECs.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Page

Introduction

This summary concludes the advances achieved within the first year of this Department of Defense award. The main goal of this project is to study the signaling mechanism downstream of VEGF receptor activation in murine breast adenocarcinoma endothelial cells, which is in part responsible for inducing angiogenic phenotypes in this tumor model. In doing so, I have found interesting differences between normal and tumor endothelial cells well worth further studying. More specifically, I have found that the response of tumor endothelial cells to VEGF is the same regardless of whether or not they have been serum-starved. Also receptor densities of VEGFR-1 and -2 differ between normal and breast tumor ECs.

Body

1. Extraction of Mouse Tumor Endothelial Cells and Mouse Liver Microvascular Endothelial Cells

In addition to extracting endothelial cells from murine mammary adenocarcinomas of TIE2-GFP+ female mice, we were initially using as control endothelial cells purchased human umbilical vein endothelial cells. Upon observations of marked differences between this normal endothelial cell group and the extracted mouse tumor endothelial cells, I decided to extract a normal endothelial cell group from mice that could serve as a more reliable control. Due to the technical difficulties of extracting endothelial cells from normal mouse mammary fat pads, I settled for extracting mouse liver microvascular endothelial cells (MLECs) and using these normal mouse endothelial cells as control cells.

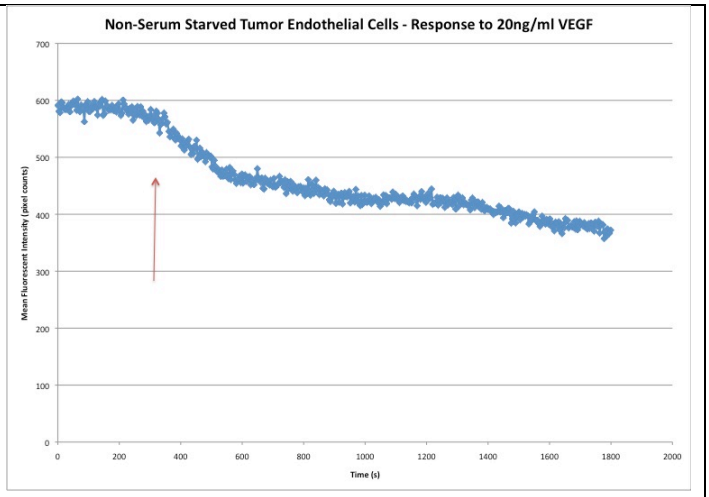
2. Experiments Using Pharmacological Reagents on Extracted Tumor Endothelial Cells

We have observed that non-serum starved mouse breast tumor endothelial cells (MBTECs) respond to vascular endothelial growth factor (VEGF) administration with an increase in intracellular calcium levels. This is interesting because healthy human umbilical vein endothelial cells (HUVECs) or MLECs in our hands do not respond to an increase in intracellular calcium levels when they have not been serum starved. This suggests that the MBTECs VEGF/calcium handling machinery has adapted to the elevated baseline levels of VEGF in the tumor media (and maintained in serum) and can still respond to alterations in VEGF even without serum starvation. Both non-serum starved and serum-starved MBTECs respond to VEGF in a similar way, and they both respond differently from healthy endothelial cells.

We have observed that non-serum starved TECs respond to VEGF administration with an increase in intracellular calcium levels (Figure 1). This is interesting because healthy ECs in our hands do not respond to an increase in intracellular calcium levels when they have not been serum starved. This suggests that the TECs VEGF/calcium handling machinery has adapted to the elevated

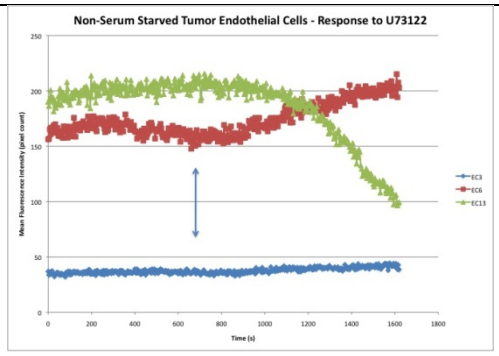
baseline levels of VEGF in the tumor media (and maintained in serum) and can still respond to alterations in VEGF even without serum starvation.

Figure 1. A representative fluorescence trace of a TEC loaded with Indo-1 AM. At the laser excitation wavelength used (750nm) Indo-1 responds to an elevation in free calcium with a decrease in fluorescence. In this case a non-serum-starved TEC (identified by its GFP fluorescence in a separate channel) responds to administration of 20 ng/ml VEGF to the medium (at the red arrow) with an increase in calcium. Non-serum-starved HUVECs do not respond in this way (data not shown).



We have also observed that non-serum starved MBTECs can respond to administration of U73122 (a PLCγ inhibitor) with an alteration in free calcium concentration (Figure 2). Some cells respond, as we predicted, with a decrease in cytoplasmic calcium concentration, indicative of our hypothesized "constitutively active" calcium handling machinery. Some cells do not respond at all, which is reasonable and interesting if one believes that TECs will be highly heterogeneous and some cells may not have this constitutive activity. However, what has been most surprising is that some cells respond to administration of U73122 with an *increase* in cytoplasmic calcium concentration. This has happened several times in several cells and warrants further investigation.

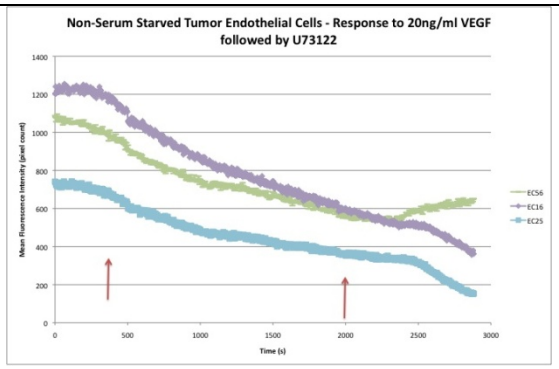
Figure 2. Representative fluorescence traces of TECs subjected to 1.12 μM of the PLCγ inhibitor U73122 (blue line). Most cells respond with a decrease in cytoplasmic calcium (red trace) or not at all (blue trace), suggesting that a heterogeneous population of cells has constitutively active calcium handling machinery. Surprisingly, some cells respond to inhibition of PLCγ with an increase in cytoplasmic calcium (green trace).



When non-serum starved TECs are subjected to 20 ng/ml VEGF followed by 1.12 μM U73122, the majority of the cells behave as expected: calcium levels increase due to VEGF administration, and this increase is attenuated by inhibition of

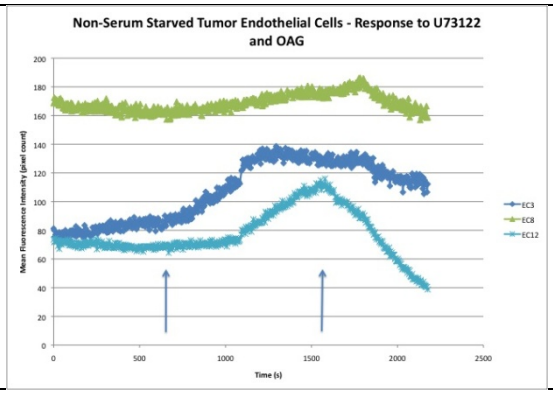
PLCγ with U73122 (green line in Figure 3). However, some cells again show the strange behavior whereby PLCγ inhibition further increased calcium levels, above those induced by VEGF (blue and purple line in Figure 3). Again, this contradicts our simple expectation that VEGF enhances cytoplasmic calcium levels via PLCγ activation.

Figure 3. Representative fluorescence traces of TECs loaded with Indo-1 AM and subjected to administration of 20 ng/ml VEGF (first red arrow) followed by 1.12 μM U73122 (second red arrow). Most TECs respond to administration of VEGF with an increase in cytoplasmic calcium which is inhibited by administration U73122 (green line). However, some TECs respond with a further increase in cytoplasmic calcium (blue and purple lines).



When non-serum starved TECs are subjected to U73122 followed by OAG (a soluble DAG analog), we see the expected decrease in cytoplasmic calcium due to U73122 inhibition of constitutively active PLCg (although with different latency times in different cells) followed by rescue of cytoplasmic calcium with OAG, a DAG analog (Figure 4). These results support our hypothesis that calcium-handling machinery in TECs is constitutively active and that it includes activation of PLCg, creation of DAG, and opening of DAG-sensitive calcium channels. Note that in these limited experiments we have not encountered cells that respond to U73122 administration with an increase in calcium and have hence not observed their response to subsequent OAG administration.

Figure 4. Representative fluorescence traces of TECs loaded with Indo-1 and subjected to administration of 1.12 μM U37122 (first blue line) followed by 100 uM OAG, a membrane-permeant DAG analog (second blue line). All the cells studied responded to U73122 with a decrease in cytoplasmic calcium (with different latency periods) followed by a rescue of cytoplasmic calcium with OAG.



3. Comparing Normal Endothelial Cells and Tumor Endothelial Cells

We have identified fundamental differences in VEGF signaling between healthy ECs (HUVECs) and tumor ECs, which suggest that one or more steps downstream of VEGFR-2 activation in tumor ECs are altered. We have also identified key differences in VEGFR-1 and VEGFR-2 density between healthy and tumor ECs, which reinforces the anomalies observed in the MBTECs VEGF signaling. Data that we have obtained from the PLC-g blockade suggest that the mechanism might be constitutively active. Interesting results have been obtained in non-serum starved cells, which are not subjected to 24 hours of serum starvation as is often done to enhance healthy EC response to VEGF (serum itself contains VEGF). In our non-serum starved cells, the cells reside in their growth medium (in the case of TECs they are kept in a medium containing a 3:2 ratio of basal endothelial cell medium supplemented with growth factors and TG1-1 cell conditioned medium) until 75 minutes before the experiment, at which time they are subjected to Pucks Saline solution, an isotonic solution of 0.68mM Ca^{2+} at a pH of 7.4).

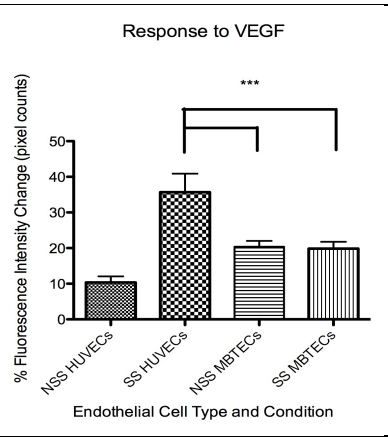
In order to analyze single cells from our imaging experiments using the two-photon microscope, we came up with a quantitative rationale to determine whether a cell has signaled after the addition of the VEGF solution. If the percent fluorescence intensity change for a single cell is equal to or greater than 5% from the average baseline fluorescence intensity level, the cell is considered as having signaled and is used in the analysis for comparison with other cell types and conditions. Otherwise, it is considered as a cell that did not signal. Based on these measurements, the total population of cells analyzed had the following percent signaling (Table 1):

TABLE 1.

Cell Type and Condition	Percentage of Cells that Signaled
NSS HUVECs	67.6
SS HUVECs	100
NSS MLECs	60
SS MLECs	100
NSS MBTECs	95
SS MBTECs	95

Our most interesting results so far are VEGF-signaling differences between normal endothelial cells and MBTECs. Typically, endothelial cells undergo 24 hours of serum starvation as is often done to enhance healthy EC response to VEGF (serum itself contains VEGF). Our results show that there is no significant difference between the signaling in MBTECs for the serum-starved and the non-serum-starved conditions, which is not the case with HUVECs and MLECs (see figures 5 and 7). There was no significant difference between the SS and NSS MBTECs groups; however, every other pair of groups analyzed were found to be significantly different from each other (Figure 5). Furthermore, the mean percent change in fluorescence intensity of both MBTECs groups was at an intermediate level between the NSS and SS HUVECs. Data was analyzed using one-variable ANOVAs with Bonferroni Correlation post-hoc tests to analyze significance between each pair of data groups.

Figure 5. Individually analyzed ECs from two-photon fluorescence intensity data were grouped into non-serum-starved (NSS) HUVECs, serum-starved (SS) HUVECs, NSS MBTECs, and SS MBTECs. This figure shows cells that signaled analyzed by their percent change mean fluorescence intensity values. ($p<0.0005$)



Another interesting finding from our imaging experiments is the fact that the dynamics of the VEGF signaling in the tumor endothelial cell groups was similar to each other in both the serum-starved and the non-serum starved groups.

Figure 6. Cells from each of the cell group and condition under study were analyzed based on the time it took for each cell to signal from the moment the VEGF solution entered the well up until the cells reached maximum signaling as indicated by minimum percent fluorescence intensity change. ($p<0.0005$)

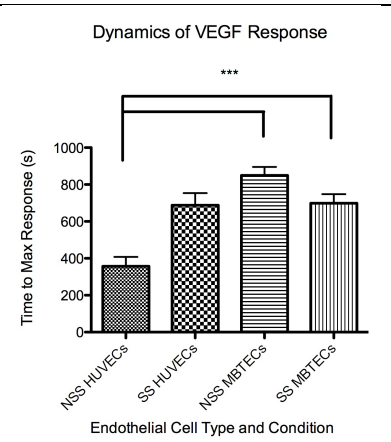
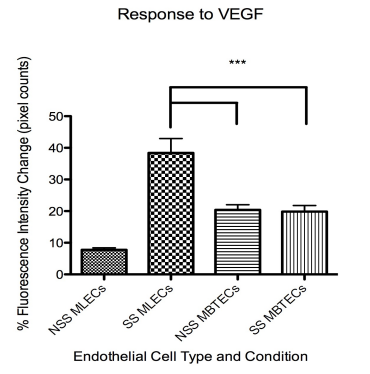


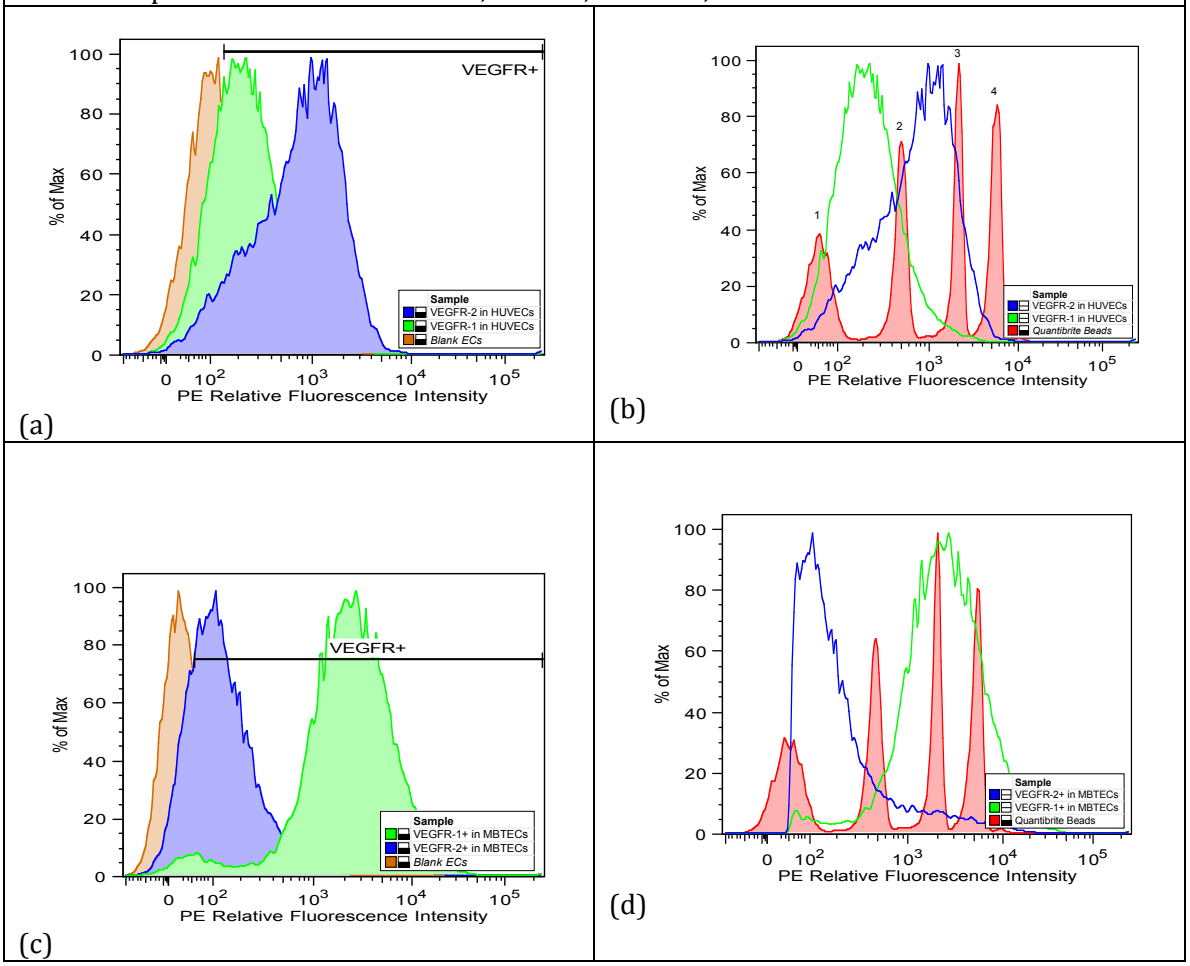
Figure 7. This figure shows similar results to the ones shown in Figure 5, but the control group is the mouse live endothelial cell group. Signaling differences between both serum-starved and non-serum starved MBTECs and the non-serum starved MLECs were found. ($p<0.0005$)



Given the strong evidence that the VEGF signaling mechanism in tumor ECs was fundamentally different from the normal ECs, I sought to investigate if the differences observed in the two-photon imaging experiments could be explained somehow by looking into the density of VEGF receptors (VEGFRs), VEGFR-1 and -2, both of which have different binding affinities to VEGF and signaling strength. I

performed several flow cytometry experiments in which I tagged different cell populations with fluorescent antibodies against VEGFR-1 and VEGFR-2 in order to compare the receptor densities and quantify the receptors per cell. The receptor density analysis performed with flow cytometry confirms that the VEGF-signaling machinery in tumor ECs has gone awry.

Figure 8. HUVECs and MBTECs were tagged with PE-labeled antibodies for either VEGFR-1 or VEGFR-2. Plots (a) and (c) show that the levels of VEGF receptor 1 and 2 are present in HUVECs and MBTECs, respectively, in significant quantities versus unlabeled ECs. Plots (b) and (d) show histograms for VEGFR-1 and VEGFR-2 groups in HUVECs and MBTECs, respectively, plotted against the quantibrite beads that contain know amounts of PE per bead. The quantibrite beads peaks (numbered on the graphs) represent the amount of PE molecules per bead as follows: 1-474, 2-5359, 3-23843, and 4-62336



Levels of VEGFR-1 are much higher and VEGFR-2 levels are lower in MBTECs when compared to HUVECs. This anomaly explains why there is attenuated signaling, as VEGFR-1 has been known to be a VEGF sink with lower signaling capacity than VEGFR-2. Figure 8 summarizes the most interesting findings from the cytometry experiments. Based on these results, the main difference lies on the fact that MBTECs have a higher amount of VEGFR-1 than normal ECs and a lower amount of VEGFR-2 than the normal counterpart. In order to make the data quantitative, I ran commercially available quantibrite beads with four groups of

known densities of dye molecules per bead, in order to be able to determine what the receptor densities were in each cell group (Figure 8b and 8d).

Key Research Accomplishments in the Past Year

1) Successfully isolated and plated two murine endothelial cell lines – one that serves as a control cell line (liver endothelial cells) and the breast adenocarcinoma endothelial cells.
2) Found key VEFG-signaling differences between normal endothelial cells and tumor endothelial cells.
3) Determined that the surface receptor densities for VEGFR-1 and VEGFR-2 are different between normal and tumor endothelial cells

Reportable Outcomes

Over the past year I have attended the following conferences with the funds from this award:

American Association for Cancer Research Annual Meeting, Orlando, FL, April 2011

Biomedical Engineering Society Annual Meeting, Hartford, CT, October 2011

I have also presented my work in a poster presentation:

Lapeira-Soto J, Madden KS, Brown E, Multiphoton Microscopy Reveals Flawed Pro-Angiogenic Signaling in Breast Tumor Endothelial Cells, Biomedical Engineering Annual Meeting, 2009

As part of my training plan, in the past year I have attended seminars and discussed topics of relevance pertaining to breast cancer with researchers at the medical center.

Conclusion

I have concluded the first year of my project in which I have successfully shown that there is a difference in signaling between healthy and breast tumor endothelial cells. The work I have performed so far has involved aspects detailed in Aim 1 and Aim 2 detailed in my Statement of Work and I have following the items I proposed in my training plan. The imaging studies performed *in vitro* laid the groundwork to start investigating these results in vivo, as was proposed in my research plan. The fact that there is no significant difference between serum-starved and non-serum starved MBTECs highlights the importance of performing these experiments in vivo, where it will not be possible to explore a non-serum starved condition.